Identification of DNA Methylation Markers for Human Breast Carcinomas Using the Methylation-sensitive Restriction Fingerprinting Technique

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Abstract

We have developed a PCR-based method, called methylation-sensitive restriction fingerprinting (MSRF), to screen changes in DNA methylation in breast carcinomas. Two hypermethylation-containing fragments, HBC-1 (for "hypermethylation in breast cancer") and HBC-2, were identified in the amplified breast tumor DNA relative to the amplified normal breast DNA of a patient. Nucleotide sequence analysis revealed no significant matches between the sequence of HBC-1 and the known sequences in the GenBank database, whereas the sequence of HBC-2 matched the upstream region of an antisense WT1 (Wilms' tumor suppressor gene) promoter. The methylation status in the breast tumor DNA from this patient was confirmed by Southern hybridization using HBC-1 and HBC-2 as probes, respectively. Further analysis showed that HBC-1 was methylated aberrantly in 90% (17 of 19 patients) of the primary breast carcinomas examined. This study demonstrates that MSRF provides a useful means for screening aberrant changes in DNA methylation during tumorigenesis. The commonly methylated fragments identified by MSRF could potentially supplement pathologival markers currently used for cancers and additionally lead to the discovery of novel methylated tumor suppressor genes.

Introduction

Abnormal changes in DNA methylation have been observed in tumor cells (for a review, see Refs. 1 and 2). These changes usually occur in CpG dinucleotides that are clustered frequently in regions of about 1—2 kb in length, called CpG islands, in or near the promoter and first exon regions of genes (3). Hypermethylation within CpG islands was found to be associated with inactivation of tumor suppressor genes, including the estrogen receptor gene (4) and the MDR gene (5) in breast carcinoma, the glutathione S-transferase gene in prostate carcinoma (6), the von Hippel-Lindau tumor suppressor gene in renal cell carcinoma (7), the RB gene in retinoblastoma (8), and the hypermethylation in cancer 1 gene (9), the cyclin-dependent kinase N2/p16 gene (10, 11), and the E-cadherin gene (12) in several types of tumor cells. In addition, aberrant hypermethylation may contribute to tumorigenesis through the C-to-T mutation at the methylated CpG dinucleotides (2). Hypomethylation was also observed in nucleotide sequences of several proto-oncogenes. Potentially, hypomethylation is linked to aberrant oncogene expression in tumor cells has yet to be established. As a further step to understanding the role of DNA methylation in tumorigenesis, abnormally hypermethylated or hypomethylated sequences occurring in the tumor genome need to be identified.

In this study, we have developed a PCR-based method, called MSRF,2 to identify abnormally methylated CpG sites in breast carcinomas. Initially, genomic DNA was digested with a 4-base restriction endonuclease known to cut bulk DNA into small fragments but rarely to cut in the CG-rich regions. The digests were treated with a second restriction endonuclease, which discriminates between methylated and unmethylated CpG sites, and then amplified by PCR with short arbitrary primers (10-mers) at low stringency. Amplified products or DNA fingerprints were resolved by high-resolution gel electrophoresis, and aberrantly methylated patterns were detected in the amplified tumor DNA relative to the amplified normal DNA of the same patient. Here we have successfully employed the technique to identify and clone genomic fragments that frequently undergo methylation changes in primary breast carcinomas.

Materials and Methods

Tissue Samples. Tumor specimens were obtained from patients with invasive breast carcinomas undergoing mastectomies or biopsies at the Ellis Fischel Cancer Center (Columbia, MO; this study has been approved by our institutional review board). The adjacent nonneoplastic breast tissue was also obtained from the same patient to serve as a control (designated as "normal"). High molecular weight DNA was extracted using standard methods.

MSRF. Genomic DNA was digested with MseI alone or digested with BstU I and MseI at 10 units per µg DNA following the conditions recommended by the supplier (New England Biolabs). The PCR reaction was performed with the digested DNA (20—100 ng) in a 20-µl volume containing a pair of primers (0.4 µM), 0.15 units of Taq DNA polymerase (Life Technologies, Inc.), 5% (v/v) DMSO, 200 µM deoxynucleotide triphosphates, and 2 µCi [α-32P] dCTP (3000 Ci/mmol; Amersham) in buffer III (30 mM Tricine (pH 8.4), 2 mM MgCl2, 5 mM β-mercaptoethanol, 0.01% (w/v) gelatin, and 0.1% (w/v) thesis) (13). The primers were as follows: BstU I, 5'-AGCGGC- CGCG; BstU I, 5'-CTCCCCACCGG; BstU I, 5'-GAGGTCGCGCGG; BstU I, 5'-AGG- GACCCCG; BstU I, 5'-GAGAGGCCGGG; BstU I, 5'-GCCGCCCCCGA; BstU I, 5'- CGGGCCGC; BstU I, 5'-GCCGCCCCCGA; and BstU I, 5'-ACCACCCCGCC. Initial denaturation was for 5 min at 94°C. DNA samples were then subjected to 30 cycles of amplification consisting of 2 min of denaturation at 94°C, 1 min of annealing at 40°C, and 2 min of extension at 72°C in a PTC-100 thermocycler (M. J. Research, Inc.). The final extension was lengthened to 10 min. The products (20 µl) of each amplification reaction were mixed with 5 µl of loading dye. Four µl of each DNA sample were size fractionated on a 4% agarose gel. DNA was transferred onto nylon membranes and subjected to autoradiography at —70°C for 24—48 h with DuPont Ultra-vision film.

Cloning and Sequencing. Target bands were excised from polyacrylamide gels, and gel fragments were recovered by centrifugation through a pinhole of a microfuge tube into a second intact microfuge tube. DNA was eluted in...
water and reamplified by 30 cycles of PCR with appropriate primers. Unmodified PCR products were ligated directly into the TA cloning vector, pCR II, and transformed into Escherichia coli competent cells following the supplier’s protocol (Invitrogen). Six to eight white colonies were selected, and plasmid DNA was isolated using minipreparation protocols (Qiagen). Double-stranded plasmid DNA was prepared and sequenced with an automated ABI model 373 sequencer (Applied Biosystems, Inc.). The resulting nucleotide sequence was compared to GenBank using the BLAST program (14).

**Southern Hybridization Analysis.** Five μg of genomic DNA were digested with MseI alone or digested with MseI and BstU I at high concentration (15 units/μg) to ensure complete digestion of both tumor and normal DNAs. The digestion products were separated on a 1.5% agarose gel and transferred to nylon membrane (Schleicher & Schuell). Filters were hybridized with a 32P-labeled HBC-1 probe (230 bp) or a 32P-labeled fragment, HBC-2.1 (119 bp; positions 21–139; see Fig. 2B). Hybridization and washing conditions were carried out essentially as described (15). Filters were exposed to Kodak BioMax film in the presence of an intensifying screen for 7 days at −70°C.

**Results and Discussion**

Several fingerprinting techniques based on arbitrarily primed PCR (16, 17) have previously been applied to identify genomic alterations (18) and differentially expressed transcripts (19) in several types of tumor cells. In the present report, we describe an improved fingerprinting technique, MSRF, to identify abnormally methylated CpG sites in the breast tumor genome. Three unique features have been implemented to preferentially analyze methylation changes within CpG islands: (a) the system used a 4-base cutter, MseI, which has previously been shown to restrict genomic DNA into small fragments (once per 140 bp) but preserve the integrity of the CpG islands (once per 1,000 bp; Ref. 20); (b) the methylation-sensitive endonuclease BstU I was used for differentiating CpG methylation between tumor and normal genomes. This endonuclease was chosen because its recognition sequence (CGCG) occurs frequently within CpG islands (once per 90 bp) but is rare in bulk DNA (one site per 5,000–10,000 bp) (20); and (c) methylated CpG islands protected from the BstU I digestion were PCR-amplified with short arbitrary primers (10-mers) attaching to the flanking homologous sequences in the genome in opposite directions. Except for primer Bs18 (see “Materials and Methods”), these primers were designed further to contain CGCG at their 3’ ends, which would enhance the chance of amplifying regions harboring the BstU I sites.

At least four conditions related to DNA methylation are distinguished by MSRF (Fig. 1A). In a condition showing no methylation (condition 1), PCR products are identified only in the MseI-digested tumor and normal DNAs (Fig. 1A, Lanes 2 and 4) but not in the MseI/BstU I-digested tumor and normal DNAs (Fig. 1A, Lanes 1 and 3). The absence of products in Lanes 1 and 3 (Fig. 1A) occurs because the given genomic sequence, which contains an internal CGCG site(s), is restricted with BstU I and cannot be amplified by PCR using appropriate primers. In condition 2, an internal CGCG site(s) for a given genomic sequence is methylated in both tumor and normal genomes and thus is protected from the BstU I digestion. Alternatively, the given genomic sequence does not contain an internal BstU I recognition sequence(s). In either situation, amplified products are present in all four lanes (Fig. 1A). An aberrant hypermethylation event (condition 3) occurs in tumor when an amplified product is observed (or shows a relative increase in band intensity) in the MseI/BstU I-digested tumor DNA (Fig. 1A, Lane 1) but not in the double-digested normal DNA (Fig. 1A, Lane 3). Amplified products are seen in the MseI-digested tumor and normal DNAs (Fig. 1A, Lanes 2 and 4). This indicates that a BstU I site(s) is specifically methylated in tumor DNA, but not in normal DNA. Hypomethylation (condition 4) is detected in tumor when a PCR product is observed in the double-

**Fig. 1.** MSRF. **A,** possible outcomes of DNA methylation changes in tumor detected by MSRF. **B,** genomic DNAs from breast tumor (T) and normal (N) tissue pairs were treated (+) with MseI. The digests were further treated (+) or untreated (−) with BstU I. Patient numbers are shown above lanes. The digests were separately amplified with primer pairs Bs7Bs10 (a), Bs1/Bs18 (b), Bs12/Bs13 (c), Bs13/Bs17 (d), and Bs11/Bs5 (e). The amplified products were size fractionated on 4.5% polyacrylamide gels. Patient numbers are shown above lanes. Molecular weight markers (100-bp ladder; Life Technologies, Inc.) are shown to the right. Arrowheads, hypermethylation-containing DNA fragments in tumor DNA.

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digested normal DNA (Fig. 1A, Lane 3), but not (or in a reduced quantity) in the double-digested tumor DNA (Fig. 1A, Lane 1).

Representative results of MRSF screening using a panel of primer pairs are shown in Fig. 1B. The majority of amplified products showed either condition 1 (no methylation) or condition 2 (normal methylation or no internal BstU I sites). Two candidate fragments containing abnormally hypermethylated BstU I sites (condition 3) in the tumor DNA were observed in Fig. 1B, b and c at ~230 and ~200 bp, respectively (Fig. 1B, arrowheads); these DNA bands were detected in the MseI/BstU I-digested tumor DNA, but not in the double-digested normal DNA. Methylation differences between amplified tumor and normal DNAs were not noted in Fig. 1B, a, d, or e. PCR products were not observed in the control reactions (no DNA template) amplified with these primer pairs at the concentration specified in the protocol.

The two hypermethylation-containing fragments (~230 and ~200 bp), now designated as HBC-1 and HBC-2, respectively, were cloned for sequencing. The fragments were found to have the expected primers at the 5’ and 3’ ends (Fig. 2, A and B, top). A BLAST search revealed no significant matches between the known sequences in the database and the sequence of HBC-1. Subsequent observation has shown that HBC-1 contains a novel expressed sequence based on a Northern hybridization analysis.4 Studies are currently underway to clone the full-length cDNA related to HBC-1. HBC-2 matched the upstream region of the antisense WT1 promoter (Wilms’ tumor suppressor gene) promoter located in the first intron on the chromosome 11p13 region; the 3’ end of HBC-2 at position 157–206 matched the published sequence of the antisense WT1 promoter at position 1–50 (GenBank accession no. S79781; Ref. 21), while the rest matched the unpublished sequence.5

We further confirmed the findings of aberrant hypermethylation in breast tumors by Southern hybridization analysis. The HBC-1 probe detected an ~800-bp band (Fig. 2A, Lanes 2 and 4) in the MseI-digested tumor and normal DNAs from patient 47. HBC-1 also detected an ~800-bp fragment in the MseI/BstU I-digested breast tumor DNA from patient 47 (Fig. 2A, Lane 1) but hybridized to a band of a smaller size (~190 bp) in the digested normal DNA of the same patient (Fig. 2A, Lane 3). This result indicates that the BstU I sites located within the ~800 bp Msel-digested fragment were protected (i.e., methylated) from restriction in the tumor DNA. The ~190-bp band detected corresponds to the BstU I-restricted fragment within the HBC-1 sequence, indicating that the two BstU I sites were unmethylated in the normal DNA. (A minor band at ~190 bp was observed at the Msel/BstU I-digested tumor DNA, which can be attributed to the presence of contaminating normal tissue in the tumor sample.) A similar Southern hybridization result was obtained when HBC-2 (an internal fragment of HBC-2 from the antisense WT1 promoter) was used as a probe (Fig. 2B, bottom). The BstU I sites located within the ~1000 bp Msel-digested fragment were found to be methylated in the digested tumor DNA (Fig. 2B, Lane 1). HBC-2 detected two fragments (~200 and ~500 bp) in the digested normal DNA (Lane 3); the ~200-bp fragment corresponds to the BstU I-digested fragment within HBC-2, indicating that the two BstU I sites were unmethylated in the normal DNA, whereas the ~500-bp fragment can be attributed to the presence of partially methylated BstU I sites in some normal cells.

We extended the methylation analysis of HBC-1 to an additional 18 breast tumor-normal DNA pairs. Fig. 3A shows representative results of four patients using MRSF. The HBC-1 fragment was detected in the amplified double-digested tumor DNA of patients 19, 25, and 65, but not in that of patient 31. HBC-1 was not noted in the double-digested normal

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4 T. H-M. Huang, unpublished data.

5 K. T. A. Malik, personal communication.
METHYLATION-SENSITIVE RESTRICTION FINGERPRINTING

Fig. 3. Representative results of methylation-sensitive restriction fingerprinting (MSRF) and Southern hybridization. A, Genomic DNA from breast tumor (T)/normal (N) tissue pairs of patients was treated (−) or untreated (+) with restriction endonucleases as indicated. The digest were amplified with a primer pair (Bsl and Bs18). The amplified products were size-fractionated on 4.5% polyacrylamide gels. Patient numbers are shown at the top of lanes. Molecular weight markers (100-bp ladder; Life Technologies, Inc.) are shown to the right. Arrowheads, the location of a hypermethylation-containing fragment HBC-1 (see explanation in Fig. 1A). B, Genomic DNA (5 μg) was treated (+) or untreated (−) with restriction endonucleases as indicated and subjected to Southern hybridization analysis using HBC-1 as a probe. T, breast tumor, N, normal breast tissue. Patient numbers are shown at the top of lanes. Molecular weight markers (100 bp ladder; Life Technologies, Inc.) are shown to the right.

DNA of these patients (Residual intensities of bands seen in the double-digested normal DNA could be due to incomplete restriction of BsrU I or low-level methylation). A parallel Southern hybridization analysis (Fig. 3B) was also conducted, showing a consistent finding of hypermethylation detected in the tumor DNA of patients 19, 25, and 65. The digested tumor DNA of patient 31 showed at least two smaller fragments (Fig. 3 B, arrowheads), ~200 and ~400 bp, that can be attributed to partially methylated or unmethylated BsrU I sites within the MseI-restricted fragment. This condition may reflect the heterogeneous nature of this primary tumor or contamination of normal cells in the tumor specimen analyzed. The methylation data based on Southern hybridization as well as patients’ clinical information are summarized in Table 1. Taken together, HBC-1 was methylated aberrantly in 90% (17 of 19 patients) of the primary breast tumors examined. Currently, we are studying more tumor samples to determine whether hypermethylation of HBC-1 is correlated with specific clinicopathological parameters of the patients with breast cancer.

Hypermethylation of HBC-2 within the antisense promoter in the intron 1 of WT1 also appeared to be common in primary breast carcinomas. We are currently extending the methylation study to the promoter and first exon regions of WT1. The results will be reported in a separate paper.

Several conditions may interfere with identification of true abnormal methylation sites from breast tumors. As discussed by others, artifacts occur in the PCR-based methodologies such as representational difference analysis (22) or differential display (23). As shown in Fig. 3A, this potential problem can be minimized by studying independent tumors and by scoring the same aberrant PCR products only consistently revealed in a group of patients. Secondly, because
CANCER (27). ND, not determined.

primary tumor specimens rather than homogeneous cell lines were present in the cancer biopsies, or vice versa. Nevertheless, the residual tissue contamination problem does not apply to detecting the gain of PCR fragments containing hypermethylated CpG sequences in tumor specimens with multicenter or multifocal lesions, in which it is not possible to obtain pure tumor cells. However, the detection of hypomethylation-containing fragments by MSRF can be impaired if contaminating normal cells are present in the tumor tissue to be tested. Because a small amount of genomic DNA (20—100ng) is applied in MSRF, one future improvement would be the use of microdissection.

Table 1 Clinical information and methylation studies of female patients with breast tumors

<table>
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<tr>
<th>Patient no.</th>
<th>Age at diagnosis (yr)</th>
<th>Histological type</th>
<th>Clinical staging</th>
<th>Methylation status of HBC-I in tumor</th>
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<tr>
<td>3</td>
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<td>+</td>
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<td>83</td>
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\( ^{+} \) well-differentiated; MD, moderately differentiated; PD, poorly differentiated; IDC, infiltrating ductal carcinoma.

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References


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